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I, Philip M. Morris, a translator residing at P.O. Box 670907, Dallas, Texas 75367 verify that I know well both the German and the English languages, that I have prepared the attached English translation of PCT Patent Application PCT/EP2004/006886 in the German language entitled "Plasmid-Free Clone of E. coli Strain DSM 6601" with the international filing date of June 25, 2004 and that the attached English translation of this document is a true and correct translation of the documents attached thereto to the best of my knowledge and belief.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of this document.

Philip M. Morris

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Applicant (*for all countries of designation except the* USA): PHARMA-ZENTRALE GMBH [DE/DE]; Loerfeldstrasse 20, 58313 Herdecke (DE)

Inventors and
Inventors/Applicants (*only for the* USA): Jorg Hacker [DE/DE]; Edith Stein-Strasse 6, 97218 Gerbrunn (DE). Tobias Oelschlaeger [DE/DE]; Mainstrasse 68, 97318 Kitzinger (DE). Sybille Oswald [DE/DE]; Burkharderstrasse 32, 97082 Wurzburg (DE). Ulrich Sonnenborn[DE/DE]; Eichenweg 6, 44799 Bochum (DE). Hans Proppert [DE/DE]; Rosenstrasse 102, 58095 Hagen (DE).

Attorney: Harmsen, Utescher; Alter Wall 55, 20457 Hamburg (DE).

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Title: PLASMID-FREE CLONE OF E. COLI STRAIN DSM 6601

[Figure 1]

Abstract: [in English]

Plasmid-Free Clone of E. coli Strain DSM 6601

The invention relates to a plasmid-free clone of E. coli strain DSM 6601, a method of its preparation and the use of the bacteria obtained in this manner as a cloning vehicle.

E. coli, a bacteria that occurs in nature, especially in the intestines of humans and animals, has long been the subject of intensive microbiological and genetic engineering research and is used in genetic engineering in particular for cloning and/or expressing certain genes and/or proteins.

Most strains of the genus Escherichia are pathogenic outside of the lumen of the bowels and generally cause infections at the affected sites. A non-pathogenic strain of the genus Escherichia coli is the strain DSM 6601 deposited in the German Collection for Microorganisms, that deviates in a few genetic features from all other E. coli strains. However, it turned out that this strain can be manipulated genetically only under difficult circumstances and partially not at all and therefore cannot be used as a simple cloning means.

E. coli DSM 6601 naturally contains two plasmids designated as pMut1 or pMut2 that have a size of 3177 and 5552 kb. These plasmids and their DNA sequences are described, e.g., in US 6, 391, 631.

Starting with the consideration that the pathogenicity and non-pathogenicity of E. coli bacteria are obviously partially controlled by their plasmids, and with the consideration that the partially occurring "genetic

resistance" of E. coli strains might also be connected to its cryptic plasmids, the invention has the basic problem of developing a plasmid-free E. coli strain that is otherwise genetically totally identical to the additional strain as regards its genomic DNA.

The above problem is solved by making available a plasmid-free clone of E. coli strain DSM 6601 and by a method of preparing such a clone.

Figure 1 shows a schematic of the method for preparing the plasmid-free clone of strain DSM 6601.

Figure 2 shows the physical map of plasmid pMut1-Tc.

Figure 3 shows the physical map of plasmid pMut2-Kn.

It turned out in the exhaustive investigations that led to the present invention that plasmid-free clones of strain DSM 6601 cannot be prepared at all with normal genetic engineering methods or can be prepared only with great difficulty so that special paths must be taken in order to generate such clones. Since the wild type of the strain has two plasmids of different sizes in addition to its genomic DNA, the elimination of these plasmids must take place in several steps that take place in part in parallel.

In order to prepare the clones in accordance with the invention the plasmids pMut1 and pMut2 occurring naturally in E. coli strain DSM 6601 are marked in accordance with the method of the invention in a first step with an antibiotic resistance. To this end the plasmids are isolated according

to traditional methods and the resistance gene inserted at the desired site. According to a preferred embodiment the resistance gene is inserted into the particular plasmid together with an expression cassette containing a promoter that can be constitutive or also inducible.

Plasmids obtained in this manner are then introduced into a suitable host according to traditional methods, e.g., the $CaCl_2$ method or electroporation where they are cloned, during which the resistance gene inserted into the particular plasmid facilitates the selection of the host cells carrying the plasmid. A host suitable for cloning the plasmid carrying a resistance gene is, e.g., the E. coli strain DH5α or E. Coli HB101.

The plasmids carrying the resistance gene are isolated and the *sac*B gene is subsequently introduced into the plasmids in order to make another marker available.

The plasmids obtained and marked in this manner are then introduced into E. coli strain DSM 6601.

After transformation the E. coli DSM 6601 are cultivated on a medium containing the antibiotic/antibiotics for which the resistance genes introduced in the preceding steps into the plasmids impart resistance.

By cultivating in such a medium, only clones grow that are resistant to the antibiotics contained in the nutrient medium. Furthermore, the bacteria lose excess genetic material since they no longer require the original plasmids pMut1 and pMut2 for their growth so that the bacteria finally

contain only the modified plasmas pMut1 and pMut2 (that contain the resistance gene/genes and the sacB gene).

In a further step the bacteria cultivated in this manner are transferred into a nutrient medium that inhibits the growth of bacteria containing the *sacB*, during which a selection pressure is exerted that substantially only permits the growth of bacteria that have lost the plasmid carrying the *sac*B gene. This can take place by cultivating the strain at 30°C in the presence of 10% saccharose, since under such conditions only those clones can replicate that have lost the plasmid carrying the *sac*B gene.

As a consequence, a plasmid-free derivative of strain DSM 6601 is obtained. It was now found that the clones in accordance with the invention, with the condition of the loss of the plasmids, did not experience any change of the genomic DNA and can surprisingly be readily used as cloning vehicles.

Thus, they can be safely used in the laboratory as host cell for the cloning and expression of a plurality of genes and proteins. Experiments with strain DSM 6601 ΔpMut1/2 have shown that it is an especially good accepter for foreign DNA when the latter is integrated into its own plasmids present in isolated form, that is, therefore its own plasmids function as cloning vectors for the foreign DNA. Furthermore, since they are derived from a non-pathogenic strain, they can be used for the treatment of disturbances of the gastrointestinal tract in animals and humans. To this end they can be transformed, if desired with foreign genes that further the

adhesion of the bacteria to the mucosa such as, e.g., adhesines that further the adhesion of the bacteria, optionally host-animal specifically, to the mucosa of, e.g., cattle and/or swine and thus hinder or prevent the growth of other pathogenic microorganisms.

The present invention will now be explained in detail with reference made to the examples.

Example 1

Modification of pMut 1

The two naturally occurring plasmids pMut1 and pMut2 of wild type isolated in accordance with the plasmid midi-prep protocol of QIAGEN (QIAGEN Plasmid Purification Handbook 12/2002, pages 16-20).

The tetracycline resistance cassette derived from vector pBR322 was selected for marking the plasmid. The associated promoter was taken from plasmid pASK75. The plasmid resulting from this cloning was designated by pKS-tetA$^{tetp/o}$. The insert tetA$^{tetp/o}$ (insert size 1 .5 kb) was cut out with restriction enzymes X*ba*I and H*ind*III. The X*ba*I /H*ind*III fragment was introduced into plasmid pMut1 via a *Nde*I restriction site.

To this end the restriction batch was purified via a column (Qu1agen, PCR purification kit) after restriction digestion of the plasmids with the appropriate enzymes and subjected to a Klenow treatment in order to form blunt ends. In order to prevent a religation of the plasmid pMut1 a

dephosphorylation of the plasmid linearized with restriction enzyme *Nde*I and treated with Klenow enzyme was carried out. Subsequently, the vector pMut1 and the insert $tetA^{tetp/o}$ were litigated and the E. coli K-12 strain DH5α transformed therewith.

In order to prepare competent cells 150 mm LB medium (Lurea-Bertani medium) was inoculated with 1.5 mm of a ÜN [? - non-standard abbreviation – overnight? – see end of paragraph] culture and agitated at 37°C until an OD_{600}=0.5. The bacterial culture was then incubated 20 minutes on ice and centrifuged 10 minutes with 4000 rpm at 4°C. The bacterial pellet was washed 3 times in sterile, ice-cold 10% glycerol, at first with 100%, then with 50% and finally with 10% of the initial volume. Finally, the washed pellet was re-suspended in 300 µl 10% glycerol, aliquoted (40 µl) and stored at -80°C. For the transformation of bacterial cells 1-2 µl plasmid DNA (1-100 ng) was mixed with 40 µl competent cells thawed on ice and incubated 5 minutes on ice. This batch was then pipetted free of air bubbles between the two electrodes of a sterile and pre-cooled 2 mm electroporation cuvette. The cuvette was dried off well and inserted into the electrode holder. After the electrical impulse had been performed at 2.5 kV, 200 Ω and 25 µF the bacterial suspension was washed out of the cuvette with 1 mm LB medium and incubated at 37°C for 1-2 hours in an agitator. The bacteria were subsequently centrifuged off and the supernatant drawn off up to 100 µl. The sediment was re-suspended in the remaining 100 µl,

plated out onto a Tc-containing selection plate and incubated ÜN [overnight?] at 37°C.

The success of the cloning was tested by minipreparation of the particular plasmid of individual bacterial clones. The plasmid pMut1 marked with the tetracycline cassette was designated as pMut1-Tc. Figure 2 shows the physical map of plasmid pMut1-Tc. It indicates the insertion site (originally a singular *Nde*I site) for the DNA fragment that imparts tetracycline resistance. This DNA fragment also contains the singular *Eco*-RI sequence suitable for clonings. Furthermore, the binding sites for the primers muta 5 and muta 6 are indicated that are suitable for the specific demonstration of this plasmid by PCR.

Example 2

Modification of pMut2

A kanamycin resistance cassette was selected for the marking of plasmid pMut2 that was derived from vector pACYC177. To this end the resistance cassette (size 1 .34 kb) was cut out of the latter with the restriction enzyme *Stu*I and introduced via a B*gl*II restriction cleavage site into plasmid pMut2. After restriction digestion of the plasmids with the appropriate enzymes the restriction batch was purified via a column (Quiagen PCR fraction kit) and plasmid pMut2 subjected to a Klenow treatment in order to form blunt ends for the cloning. In order to avoid a religation of plasmid

pMut2 a dephosphorylation linearized with restriction enzyme B*gl*II and treated with Klenow enzyme was carried out. Subsequently, vector pMut2 and the kanamycin cassette were litigated and the E. coli K-12 strain DH5α transformed as described in example 1. The transformation was plated out onto Kn-containing LB agar plates. The success of the cloning was tested by minipreparation of plasmid DNA of individual bacterial clones. Plasmid pMut2 marked with the kanamycin cassette was designated as pMut2-Kn.

Figure 3 shows the physical map of plasma pMut2-Kn. The insertion site (originally a *Bgl*II site) for the kanamycin resistance cassette as well as the singular *Eco*-RI sequence suitable as cloning site are sketched in. Muta 7 to Muta 10 characterize those areas complementary to the sequences of the primers that are suitable for the specific demonstration of this plasmid by PCR.

Example 3

Introduction of the *sacB* gene

In order to introduce the *sacB* gene (coded for a Levan saccharose) into the plasmids pMut1-Tc and pMut2-Kn, the particular singular *Eco*-RI restriction cleavage site was selected in both plasmids. The *sacB* gene (size 2 .6 kb) was isolated from the plasmid pCVD442 with the restriction enzyme *Pst*I. In order to prepare the vectors pMut1-Tc and pMut2-Kn, these plasmids were linearized with *Eco*-RI and subsequently submitted to a

Klenow treatment and dephosphorylated in order to form blunt ends. The insert *sacB* was purified with *Pst*I after the restriction digestion and treated with Klenow enzyme. The linearized vectors and the insert were ligated and the E. coli K-12 strain DH5α, that was rendered competent as described in example 1, was transformed therewith. The success of the cloning was checked by minipreparation of plasmid DNA of individual bacterial clones and subsequent restriction analysis. The plasmids pMut1-Tc and pMut2-Kn marked with the *sacB* gene were designated as pMut1-TcSac and pMut2-KnSac.

Example 4

Preparation of a plasmid-free clone of E. coli strain DSM 6601

At first the plasmid pMut1-TcSac was transformed by electroporation into this strain as described in example 1. After the electroporation in order to transfer plasmid pMut1-TcSac into strain DSM 6601 the batch was plated out onto LB plates containing Tc (50 µg/ml). The tetracycline-resistant bacterial clones obtained were checked for the possession of plasmid pMut1-TcSac and the associated loss of the naturally occurring plasmid pMut1. The loss of plasmid pMut1 was demonstrated after preparation of the plasmid DNA and restriction digestion of the same with the enzyme *Eco*-RI after electrophoretic separation of the linearized plasmids by the lack of the DNA bands representing pMut1.

Subsequently, one of these clones was attracted overnight in LB medium with 10% saccharose at 30°C and then plated out onto LB plates with 10% saccharose (LB medium consists of 10 g peptone from casein, 5 g yeast extract and 5 g sodium chloride per 1 l distilled water). Saccharose-containing LB medium was prepared in that a part of a sterilely filtered, 50% (wt./vol.) saccharose stock solution was added to the autoclaved medium after it had cooled off to 45°C up to an end concentration of 10%). The plates were incubated at 30°C. Under these conditions only those clones can replicate that no longer express *sacB*, that is, they have lost the plasmid carrying the *sacB* gene. The resulting strain DSM 6601ΔpMut1 was checked for the loss of plasmid pMut1-TcSac.

Then, plasmid pMut2-KnSac was introduced by electroporation into strain DSM 6601ΔpMut1.

After the electroporation in order to transfer plasmid pMut2-TcSac into strain DSM 6601ΔpMut1, the batch was plated out onto LB plates containing Kn (50 μg/ml). The kanamycin-resistant bacterial clones obtained were checked for the possession of plasmid pMut2-KnSac and the associated loss of the naturally occurring plasmid pMut2. Subsequently, one of these clones was attracted overnight in LB medium with 10% saccharose at 30°C and then plated out onto LB plates with 10% saccharose. The plates were incubated at 30°C. The resulting strain DSM 6601 ΔpMut1/2 was checked for the loss of plasmid pMut2-KnSac.

Furthermore, a Pulsfeld gel electrophoresis of the plasmid-free strain DSM 6601 ΔpMut1/2 was carried out in order to exclude any chromosomal modifications of the strain. It was determined that no changes could be demonstrated and further examinations showed that the plasmid-free clone displayed no morphological, biochemical or fermentative changes.